STUDIES IN THE PHYSIOLOGY OF SPERMATOZOA.[1]

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I. Introduction.

The changes in the physiological condition of the spermatozoon, from the time it is extruded from the genitalia of the male until it "undergoes the transformation into a nucleus" (Loeb, J., 1913, p. 306) in the protoplasm of the egg are dependent in rate upon environmental conditions. The germ cells of most marine invertebrates are extruded into sea water, and fertilization of the egg by the sperm there follows. The environment, sea water—or sea water modified by the excretions of the egg or of the sperm—must therefore be studied in order to understand the variations in the physiological condition of spermatozoa that have often been observed.

This investigation had its beginning in an attempt to understand seemingly contradictory effects of sea water that had contained the eggs of the sea urchin,[1] *Arbacia punctulata*, upon the activity, the length of life and the "fertilizing power" of the spermatozoa of the same species. For interesting me in these phenomena, and for invaluable aid in this attempt at their solution, I am indebted to Dr. F. R. Lillie.

The experimental work was carried on during the summers of 1915 and 1916 at the Marine Biological Laboratory at Woods Hole, Massachusetts. During that time the behavior of the germ cells of other marine invertebrates were sufficiently observed to suggest that the relations that are hereafter reported for *Arbacia* are not highly specific.

Material and Methods.

The sperm of the sea urchin, *Arbacia punctulata*, are shed from the genital pores, if the peristome is cut, and the urchin placed aboral side down. The sperm may then be collected in a clean dry watch glass, and diluted to any concentration by the admixture of sea water. The concentration of the sperm suspension records the extent of dilution of the sperm. A one per cent. suspension is one in which one drop of sperm is added to 99 drops of sea water or of sea water that has suffered a definite modification.

The relative "fertilizing power" of sperm that had been sub-

[1] Such sea water is for convenience called egg water.

jected to different environments was determined by adding such sperm in identical concentration to the ripe eggs of the same species in sea water at different times. The average number of eggs that subsequently developed was estimated by counting at least a hundred eggs at about the four cell stage.[1]

In tabulating the experiments that are reported (in Tables I., II. and III. the concentration of spermatozoa was the variable environmental condition) the concentration of spermatozoa in the suspension is recorded in a column at the extreme left. The variable environmental conditions that obtained in the different suspensions are recorded in a legend over the columns representing the percentage of eggs that were fertilized when a definite number of drops of the sperm suspension (the number of drops of sperm is reported either in the legend or in a column at the extreme right) were added to a constant quantity of eggs in a given amount of sea water at the intervals noted.

The effects of change in environmental condition upon sperm were under investigation. Since the influence of change in environmental condition upon eggs and upon sperm are not dissimilar (Loeb, J., 1913, Robertson, T. B., 1912) the variation in the physiological condition of eggs (Gemmill, 1900; Vernon, 1899; Loeb, J., 1913; Goldfarb, A. J., 1917) was eliminated by always inseminating in sea water.

For the same reason, the eggs of but one female were used in each series of inseminations. The fertilizing power of different sperm suspensions were in this way tested. The eggs were obtained by straining the cut up ovaries through cheese cloth into sea water, and subsequently washing the eggs by decanting the supernatant fluid. Eggs were never used after they had been in sea water for more than six hours. The forceps that were employed in removing the ovaries were never used for any other purpose. If a male had previously been opened, the hands and the scissors with which the peristome had been cut were rinsed in fresh water. A control of unfertilized eggs was always kept, but no contamination was ever observed.

The failure of sperm to fertilize ripe eggs may be employed as

[1] See Lillie, F. R., 1915; Fuchs, H. M., 1915, for a detailed description of this procedure.

an indication of death only if accompanied by some other observation such as the dissolution of the protoplasm that follows the death of spermatozoa. This is necessary, since a decrease in the activity of spermatozoa may also decrease their fertilizing power. But sperm that are non-motile can be reactivated, and until reactivation is no longer possible, sperm can not be considered dead.

The rate of movement of spermatozoa cannot, however, be observed with any degree of accuracy. While it is not difficult to distinguish between a very motile, a fairly motile and a non-motile sperm suspension, more delicate fluctuations in spermatozoön activity cannot easily be observed. As in other cells a delicate indicator of the degree of activity is afforded by the measurement of the oxygen consumption (Loeb and Wasteneys, 1912; Warburg, 1910) the heat production (Meyerhoff, 1911) or the carbon dioxide production of a sperm suspension. The carbon dioxide production of sperm suspensions of different concentration has been measured.

The activity of spermatozoa, as will presently appear, is effected by changes in either the temperature, the osmotic pressure or the hydrogen ion concentration of sea water. Since even the carbon dioxide produced by spermatozoa is sufficient appreciably to change the hydrogen ion concentration of a 0.005 per cent. suspension, since the activity of spermatozoa is a function of the hydrogen ion concentration, and since the fertilizing power of a sperm suspension is related to the activity of the spermatozoa, the careful control of the environment becomes a necessity.

Sea Water as Environment.

The environment of the spermatozoa of the sea urchin is sea water. A variable in sea water that is known to effect the activity of spermatozoa is the hydrogen ion concentration. The concentration by weight of hydrogen ions in sea water is approximately 0.00000001 N or 1×10^{-8} N (Palitzsch, Sven, 1912). In a neutral solution there are, by definition, as many hydrogen as hydroxyl ions. The concentration of hydrogen ions in a neutral solution is 1×10^{-7} N or 10×10^{-8} N. There are therefore in the neighborhood of ten times as many hydrogen ions

in a neutral solution as there are in sea water and ten times as many hydroxyl ions in sea water as there are in a neutral solution. Since the acidity or the alkalinity of a solution is measured in terms of its concentration in hydrogen ions, sea water is appreciably alkaline. The hydrogen ion concentration is often expressed as the negative logarithm to the base 10. This is called the hydrogen potential (Ph) (Sörensen, S. P. L., 1909). The hydrogen potential of sea water is therefore Ph 8. During the months of July and August, 1916, the sea water at the Marine Biological Laboratory at Woods Hole only varied between Ph 7.95 and Ph 8.15.

The hydrogen ion concentration of sea water was measured by colorimetric comparison with solutions of borates and phosphates. These were always standardized with a concentration cell. The indicators phenolphthalein, naphtolphthalein and neutral red satisfactorily covered the range investigated. Corrections for the effect of salts upon these indicators have been determined (Sörenson & Palitszch, 1910, 1913; Palitszch, 1911) and were employed.

The hydrogen ion concentration of "sea water is fully determined by (1) the tension of carbonic acid, (2) the concentration of water, or salinity, and (3) the temperature. This relationship suggests a method of determining the carbon dioxide tension of sea water" (Henderson, L. J., and Cohn, E. J., 1916, p. 620), for the effect of salinity upon the hydrogen ion concentration has been found to be very small (Henderson, L. J., and Cohn, E. J., 1916; McClendon, Gault and Mulholland, 1917). The effect of the temperature has been determined (Henderson, L. J., and Cohn, E. J., 1916; McClendon, Gault and Mulholland, 1917), and the relation between the hydrogen ion concentration and the carbon dioxide tension of sea water at a temperature of 20° C. has been reported by Henderson & Cohn (1916); at a temperature of 30° C. by McClendon (1916 and 1917). The measurement of the carbon dioxide tension of sea water (that is the partial pressure of the gas that is in equilibrium with sea water containing a definite concentration of carbon dioxide) can therefore be made with great accuracy and great rapidity. The carbon dioxide tension is recorded in terms of the number of millimeters of

mercury that represents the partial pressure of the gas in the atmosphere at a total pressure of 760 millimeters, and a temperature of 0° C.

The change in the carbon dioxide concentration of sea water is not proportional to the change in the carbon dioxide tension, for increase in the carbon dioxide tension is correlated with a change in the equilibrium between the normal carbonates and bicarbonates in sea water. It has been calculated (Henderson, L. J., and Cohn, E. J.) that the former are converted into the latter at exactly the tensions of carbon dioxide that obtain in the ocean. At tensions of carbon dioxide greater than these an increase in tension may, as a first approximation in determining the carbon dioxide concentration, be considered as an increase in free carbonic acid. A "conversion table" for determining the carbon dioxide concentration (or content) of sea water has been published by McClendon (1917). The measurements of the total carbon dioxide concentration upon which this "conversion table" is based are not reported. Exact data defining the relation between the hydrogen ion concentration and the carbon dioxide concentration of sea water are therefore still unknown.

II. Environmental Conditions that Affect the Activity of Spermatozoa.

That "all the phenomena connected with the origin and death of the spermatozoön seem to be in accordance with the view, that its motion is essential to its function" (Newport, G., 1853, p. 261) was the opinion of the early investigators of the rôle of the sperm in fertilization. Indeed so completely was the movement of the "spermatic animalcules" found to depend upon temperature (Spallanzani, quoted from Newport, 1851, p. 235) (Prevost and Dumas, 1824) (Newport, G., 1851); osmotic pressure (Koelliker, A., 1856) and hydrogen ion concentration (Koelliker, A., 1856) that the observed activity of these cells was for a time supposed to be due to Brownian movements.

"Es fällt somit die Theorie, die Bewegung der Samenfäden sei willkürliche thierische Bewegung, haltlos zusammen. Welche physikalischen Kräfte aber dieses Phänomen erzeugen mögen, ist noch völlig dunkel. Ja wir können noch nicht einmal mit Bestimmtheit behaupten, obwohl diess wahrscheinlich ist, dass

die Samenfäden auch im Organismus, im Hoden oder in den weiblichen Genitalien sich bewegen, es kann Niemand mit Bestimmtheit widerlegen, dass nicht etwa diese Bewegungen erst in den aus dem Organismus entfernten Objecten unter dem Mikroskop, als ein Analogon der Brown'schen Molecularbewegung entstehen, sei es durch Verdunstung oder irgend eine andere physikalische Ursache. Es ist mehr als wahrscheinlich, dass die Bewegungen wenigstens in einer physikalischen Wechselwirkung zwischen Flüssigkeit und Samenfäden begründet sind, wofür schon die ausserordentliche Abhängigkeit der Bewegungen von der Concentration und Beschaffenheit der Flüssigkeit, ferner vor Allem die Abänderung der Bewegungsacte durch Zusatz von Wasser, die Abhängigkeit der Art der Bewegung von der Form der Samenfäden der verschiedenen Thiere spricht, Umstände, welche auch auf andere Weise als durch einfache Adhäsionsverhältnisse, Vermehrung und Verminderung des Widerstandes zu wirken scheinen" (Koelliker, A., 1856, p. 202. Quotation from Funke im Lehrbuch der Physiol. von Günther, Bd. II., Abth. IV., 1853, p. 1027).

Dissenting from this position Newport "regarded this motion as being only the visible indication of a peculiar force, or form of vitality, in the impregnating agent, the spermatozoön, by which it is destined to arrive at, and is to expend on the object to be fecundated, and the effect of which is to strengthen, to augment, and possibly also to modify the nature of the formative changes, which are going on in the yet unimpregnated egg, per se; but which will subside, and soon entirely cease, if not reinforced through the agency of the spermatozoön" (Newport, G., 1853, p. 260). And again: "Whatever be the relation of this motion to its peculiar faculty, it is evident that motion is intimately associated with, and dependent on, its material composition and structural development" (Newport, G., 1853, p. 261).

Temperature.

The environmental conditions that effect the behavior of the spermatozoön were therefore abundantly and carefully observed by early investigators. "Spallanzani found that the fluid of the fœtid terrestrial toad (*Bufo calamita?*) at a high temperature of

the season, 70° F. to 73° F., at which this species spawns in Italy, had lost its fecundatory influence at the end of six hours; but that in the temperature of an ice house, 40° F., it retained its efficacy for 25 hours" (Newport, G., 1853, p. 235). Observations of this kind were repeated by Prevost and Dumas (1824) and Newport (1851 and 1853) who wrote: "The general conclusion which seems to be deducible from a comparison of the observations of Spallanzani and of Prevost and Dumas, with those by myself, in regard to the tail-less Amphibia, is, that . . . the vitality of the spermatozoön, and the duration of its fecundatory power, are in a ratio inverse to that of an increase of temperature in the surrounding medium" (Newport, G., 1853, p. 237).

Hydrogen Ion Concentration.

Nor was temperature the only environmental factor that was known to effect the physiological condition of the spermatozoön. In a classic paper, "Physiologische Studien über die Samenflüssigkeit" (Koelliker, A., 1856), Koelliker demonstrated that the effect of substances supposedly "harmful" to the activity of the sperm, disappeared if the solutions were first made isotonic and isohydric with the suspension. He understood that a slightly acid solution might inhibit the activity of the sperm without killing them, and that reactivation followed upon neutralization of the acidity. He observed that if KOH, Na_2HPO_4 or blood were added to suspensions of paralyzed spermatozoa motility was recovered.

That the degree of activity is a function of the hydrogen ion concentration of the sperm suspension has been confirmed by subsequent investigations. In 1907 Günther (Günther, G., 1907) showed that not only could sperm be reactivated by a decrease in the hydrogen ion concentration, but also that they could be inactivated by an increase in the hydrogen ion concentration. He noticed that if a weak electric current is passed through a mammalian sperm suspension the sperm congregate at the positive pole and are there inactive. The hydrogen ion concentration is greatest at the positive pole. If the current is reversed sperm that have been inactive at the positive pole recover their activity and accumulate at the other

end of the suspension; now become the positive pole. There they are again inactive. In the more alkaline part of the suspension the sperm are extremely active. Frog sperm (Lillie, R. S., 1903) and Echinid sperm (Gray, J., 1915) behave in a similar manner. This phenomenon does not occur if sperm are first inactivated by an increase in the hydrogen ion concentration of the suspension (Gray, J., 1915).

Oxygen and Carbon Dioxide Concentration.

Similar in kind is the observation of Buller (Buller, A. H., 1902) that when a bubble of oxygen is incorporated in a suspension of *Echinus* sperm those in the immediate vicinity of the oxygen remain active after the sperm at a greater distance "have all come to rest from want of oxygen." Between the active sperm and the inactive sperm there is a zone "in which there are comparatively very few spermatozoa." The active sperm gradually traverse the clear zone "and collect on the inner edge of the zone" (of inactive sperm) "upon reaching which they cease to move. A ring of thickly placed, dead spermatozoa thus arises" (Buller, A. H., 1902, p. 158). The spermatozoa were not "dead" but merely inactive in a region of low oxygen and of high carbon dioxide concentration.

F. R. Lillie has observed the converse phenomenon. He injected a drop of sea water with a carbon dioxide concentration of approximately 1 per cent. into a suspension of *Nereis* sperm (the sperm of *Arbacia* are not so sensitive to carbon dioxide but "the reactions of *Arbacia* spermatozoa are essentially the same in principle as those of *Nereis*") (Lillie, F. R., 1913, p. 546) and noticed that a ring of sperm is formed at a definite concentration of carbonic acid. This ring is separated by a clear zone from the active sperm in the rest of the suspension. "If the external edge of the clear zone be carefully observed, the spermatozoa can be seen to detach themselves one by one from the general suspension and pass straight over to the ring" (Lillie, F. R., 1913, p. 535).

It is apparent that increase in the hydrogen ion or in the carbon dioxide concentration or decrease in the oxygen concentration[1] decreases the activity of spermatozoa, while de-

[1] The observations of Drzewina, A., and Bohn, G. (Drzewina, A., and Bohn, G., 1912) upon the effects of lack of oxygen upon the length of life of spermatozoa will be considered in another place.

crease in the hydrogen ion or carbon dioxide concentration or increase in the oxygen concentration increases the activity of spermatozoa. Three explanations of the configuration that appears when spermatozoa are subjected to such a gradient in carbon dioxide as has been described have been suggested; (*a*) that spermatozoa are activated in certain concentrations of carbon dioxide; (*b*) that spermatozoa are positively chemotactic or chemotropic to carbon dioxide; (*c*) that the accumulation of spermatozoa at a certain concentration of carbon dioxide is brought about by their inactivity in that concentration of carbon dioxide.

While it is not inconceivable that spermatozoa are activated in certain concentrations of carbon dioxide, no evidence of such a primary stimulation of spermatozoa has ever been observed or reported.

The circumstances which led to the formulation of the second hypothesis are quite intricate and will require an historical introduction if the problem is to be understood. Ever since Pfeffer "demonstrated the importance of the part played by chemotactic stimuli in causing the spermatozoa of liverworts, mosses, ferns, etc., to approach the oöspheres" (quoted from Buller, A. H., 1902, p. 145) biologists have tacitly assumed or attempted to demonstrate that this chemotactic phenomenon is general in fertilization not only in plants[1] but in animals. In 1895 Bergh suggested that "the spermatozoa collect around the ripe eggs, probably attracted by a special substance "(quoted from Buller, A. H., 1902, p. 146). Three years later Massart (Massart, J., 1888) demonstrated that the spermatozoa of the frog were positively thigmotactic to glass. He was, however, unable to demonstrate chemotaxis. This observation had previously been made by Dewitz (Dewitz, J., 1886). Massart also maintained that spermatozoa were positively thigmotactic to agar and gell (Massart, J., 1888) especially that of the egg (Massart, J., 1889).

[1] There is some doubt that chemotaxis is a general phenomenon in the fertilization of plants. To a recent study of the "Physiology of Fucus Spermatozoids" the following summary is appended. "Using the Pfeffer capillary tube method of determining chemotaxy, it was found that certain acids cause collection of *Fucus* spermatozoids. It is suggested that this may be explained as due to toxicity and not chemotaxy" (Robbins, W. J., 1916, p. 130).

In 1900 Buller observed the "agglutination" or "cluster formation" of *Arbacia* sperm in water that had contained eggs of the same species and suggested that "a tactile stimulus appears to play a part in the phenomenon" (Buller, A. H., 1900, p. 387). As the "aggregation" of sperm by carbon dioxide has been supposed to be due to chemotaxis, so also has the "agglutination" of sperm by "egg water" (de Meyer, J., 1911, Lillie, F. R., 1913; Glaser, O., 1914). The phenomenon of "agglutination" will be discussed in another place. The configuration of *Arbacia* sperm in "agglutination" and of *Nereis* sperm in "aggregation" is not dissimilar (Lillie, F. R., 1913).

In 1902 Buller described the accumulation of inactive sperm in an oxygen gradient. This has been characterized as the converse of Lillie's "ring formation" in a gradient of carbon dioxide (p. 12). Buller explained the phenomenon as the result of the differential activity of spermatozoa in the gradient. He was, however, unable to account for the "clear zone" that occurs in the configuration. In consequence subsequent workers have had recourse to the accessory hypotheses that have been discussed. While there is no a priori reason for believing either that spermatozoa are not activated in certain concentrations of carbon dioxide or that they are not chemotactic to carbon dioxide, it should be pointed out that no positive evidence for either assumption has ever been demonstrated.[1]

III. The Relation between the Activity and the Longevity of Spermatozoa.

Increase in the activity of spermatozoa leads to a decrease in the length of time during which spermatozoa exhibit activity. This was observed by Koelliker, who remarked, regarding the effect of the alkali salts of carbonic acid, that they behaved much as the caustic alkalies in that: "Sie erregen die Samenfäden lebhaft, doch dauert deren Bewegung nicht lange" (Koelliker, A., 1856, p. 239). Koelliker emphasized that this activation oc-

[1] "Dewitz, Buller, and the writer have vainly tried to prove the existence of a positive chemotropism of spermatozoa to eggs of the same species" (Loeb, J., 1916, p. 93).

curred only in weakly alkaline solution, since sperm were "injured" in more concentrated alkaline solution.

From these and other observations Koelliker came to believe that the nourishment and therefore the length of life of the spermatozoön after it is liberated from the testes is dependent only upon the material of which it is constituted. "Eher wäre daran zu denken, ob nicht vielleicht die Körper der Samenfäden sich zu den Fäden selbst verhalten, wie eine Zelle zu ihren Wimperhaaren, und dieselben aus dem in ihnen enthaltenen reichlichern Material ernähren, eine Vermuthung, die jetzt, wo ich zeigen kann, dass die Samenfäden aus den Kernen der Samenzellen sich bilden, wohl ausgesprochen werden darf. Zu erforschen ist auch noch, ob die Samenfäden bei ihren Bewegungen elektrische Ströme entwickeln, und ob sie nicht, so lange sie sich bewegen, CO_2 abgeben, während sie O aufnehmen, Verhältnisse, über die ich vielleicht später berichten kann" (Koelliker, A., 1856, p. 245).

On the basis of observations of a quite similar kind Gemmill (1900) came to exactly the opposite conclusion. He observed that "the term of vitality of spermatozoa varies according to the degree of their admixture with sea water" (Gemmill, J. F., 1900, p. 170) and correctly concluded that "mixing with seawater stimulates the activity of movement of the spermatozoa, which become the more active the better they are mixed with pure sea water. Under these circumstances, their store of energy will be the sooner exhausted" (Gemmill, J. F. 1900, pl. 169). Gemmill was of the opinion, however, that if sperm lived longer in more concentrated suspensions it was because they there received more nourishment, for he goes on to say: "On comparing the movements of spermatozoa in different mixtures, one finds that the difference of activity is not sufficiently marked to account for the very early loss of vitality of spermatozoa in the weaker mixtures simply in terms of exhaustion of energy. I am inclined to believe that the other factor above noted, namely, the dilution of the nutritive medium by the addition of sea water, is the more important cause. An interesting sidelight on this question is afforded by some facts which will be given later regarding the keeping alive of spermatozoa by artificial nutri-

tion" (Gemmill, J. F., 1900, p. 171).[1] An experiment similar to those performed by Gemmill is reported (Table I., Experiment 28). The "term of vitality" of spermatozoa was determined by testing their fertilizing power. As in Gemmill's experiments the sperm in the most concentrated suspensions lived for the longest time.

TABLE I.

EXPERIMENT 28.

The Length of Life, as Measured by the Fertilizing Power, of Sperm Suspensions of Different Concentration.[2]

Date.	Time of Insemination.	Age of Sperm.		1	2	3	4
				Concentration of Sperm Suspensions.			
				4%.	1%.	0.5%.	0.25%.
		Hrs.	Min.	Percentage of Eggs Fertilized when 1 Drop of Sperm Added to 5 Drops of Eggs in 10 c.c. of Sea Water at Intervals as Noted.			
July 25..	12:20 A.M.	0	0				
" " ..	2:30 P.M.	14	10	100	98	67	10
" 26..	12:00 M.	23	40	100	98	15	0
" 27..	11:20 A.M.	47	00	100	0	0	0
" 28..	12:15 P.M.	71	55	98	0		
" 29..	8:20 A.M.	92	00	85			

The measurement of the total carbon dioxide production in sperm suspensions of different concentrations, which will now be reported, has however made untenable the position of Gemmill that the "exhaustion of energy" is not sufficient to account for the "loss of vitality of spermatozoa."

The Measurement of the Total Carbon Dioxide Production of Sperm Suspensions of Different Concentration.

The hydrogen potential of sperm suspensions of varying concentration was measured as a function of time.

In measuring the hydrogen potential of sperm suspensions

[1] The so-called "artificial nutrition" was brought about by adding beef broth to sea water. This increases the hydrogen ion concentration of the suspension and decreases the activity of spermatozoa. The evidence for these statements will be found in another place in this paper.

[2] The sea water in this experiment was sterilized in order to prevent the bacterial contamination which otherwise occurs when sperm suspensions are kept for so long a time (Gorham, F. R., and Tower, R. W., 1902). Erlenmeyer flasks were used in the experiment. On the third day suspension 1 was still relatively free from bacteria. The spermatozoa appeared healthy and were not motile.

no such accuracy can be attained as in the measurement of the hydrogen potential of sea water, since the cloudiness of the more concentrated suspensions makes the colorimetric determination difficult and since the intensity of the indicators changes more rapidly in the presence of sperm. This is probably due to the

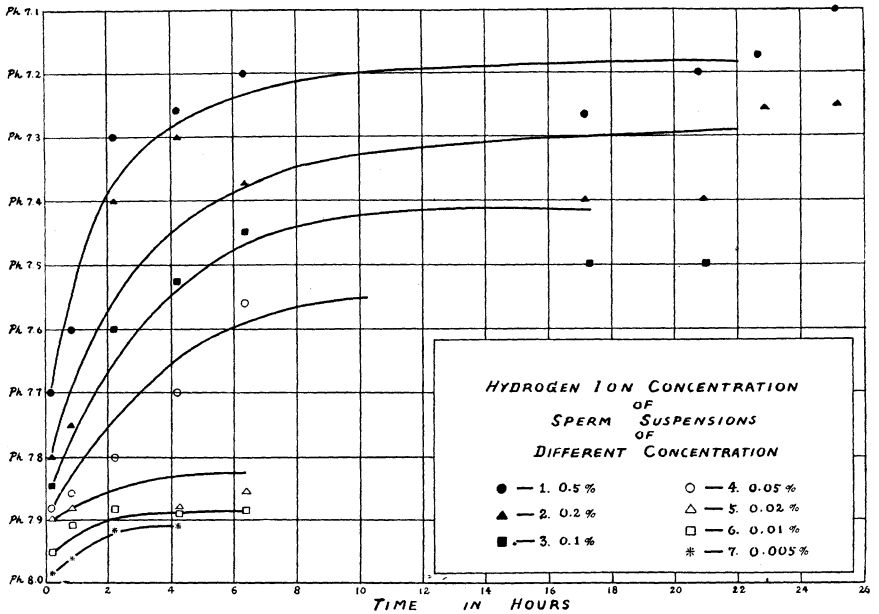

DIAGRAM I.

penetration of the indicators into the cells and observations suggest that this depends upon the physiological condition of the sperm. These effects can, however, be prevented from interfering with the measurement of the hydrogen potential of the suspensions if the measurements are made immediately after the indicators are added. If these precautions are observed, a sufficient accuracy for biological purposes can be attained.

The data obtained in this way are in Table II. and are graphically represented in Diagram I. The ordinate represents the hydrogen potential of the suspensions; the abscissa, the time in hours. The concentration of sperm in each suspension is recorded in the accompanying tabulation. Since the increase in hydrogen ion concentration is due to the carbon dioxide produced

by the spermatozoa, and since the rate of carbon dioxide production is, in turn, a function of the hydrogen ion concentration of the suspension, in all measurable concentrations the carbon dioxide production of sperm suspensions falls off with time.

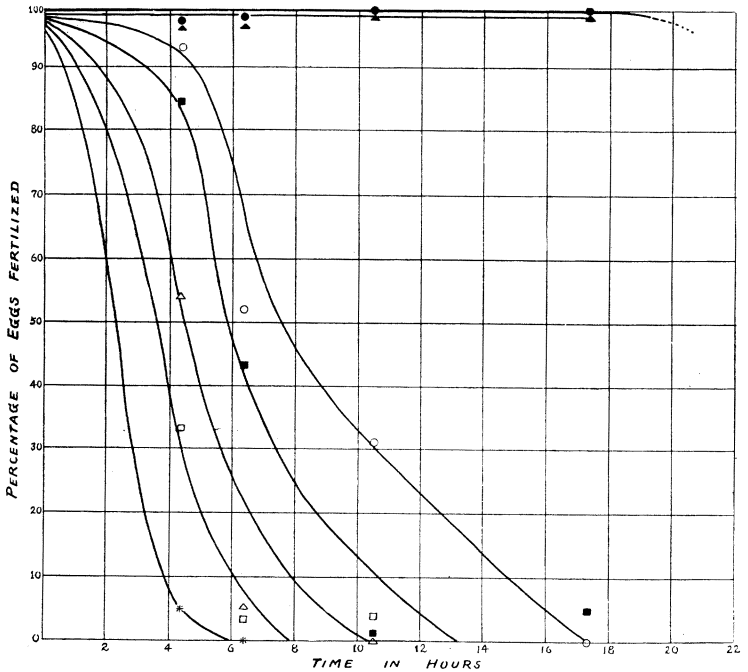

DIAGRAM II.

"The term of vitality of spermatozoa varies according to the degree of their admixture with sea water" (Gemmill, J. F., 1900, p. 170). In the most concentrated suspensions the spermatozoa live for the longest time. The length of life of the different suspensions as computed from the per cent. of eggs that were fertilized by identical concentrations of sperm under identical experimental conditions is recorded in Table III. and graphically represented in Diagram II.

The concentration of carbon dioxide, like the tension of carbon dioxide with which it is in equilibrium, in any one sample of sea water at any one temperature is completely determined by the hydrogen potential. As an approximation, sufficient for the

calculation of the carbon dioxide produced by spermatozoa in the above experiment, an increase in carbon dioxide tension (as measured by change in hydrogen potential) may be considered as an increase in free carbonic acid.

TABLE II.

HYDROGEN POTENTIALS OF SPERM SUSPENSIONS.

Age of Sperm Suspensions.		1	2	3	4	5	6	7
		Concentration of the Sperm Suspensions.						
		0.5%.	0.2%.	0.1%.	0.05%.	0.02%.	0.01%.	0.005%.
Hours.	Minutes	Hydrogen Potentials of Sperm Suspensions.						
	5	7.70	7.80	7.85	7.88	7.90	7.95	7.98
	50	7.60	7.75	7.80	7.86	7.88	7.91	7.96
2	20	7.30	7.40	7.60	7.80		7.88	7.92
4	20	7.26	7.30	7.53	7.70	7.87	7.87	7.91
6	20	7.20	7.37	7.45	7.56	7.86	7.88	7.95
17	20	7.26	7.40	7.50	7.74	7.90	7.98	8.04
20	50	7.20	7.40	7.50				
22	50	7.17	7.26	7.40	(7.74)	(7.88)	(7.91)	(7.98)
24	50	7.10	7.26	7.40				

TABLE III.

LENGTH OF LIFE OF SPERM SUSPENSIONS.

Age of Sperm Suspensions.		1	2	3	4	5	6	7
		Concentration of the Sperm Suspensions.						
		0.5%.	0.2%.	0.1%.	0.05%.	0.02%.	0.01%.	0.005%.
Hours.	Minutes.	Percentage of Eggs that Were Fertilized in Sea Water by Identical Concentrations of Sperm.						
4	20	98	98	85	94	54	34	6
6	20	99	97	43	52	6	4	0
10	20	100	100	2	31	0	4	7
17	20	100	100	5	0	0	0	0
		Approximate length of life of spermatozoa as computed from their failure longer to fertilize eggs of the same species.						
		17+	17+	17+	10+	6+	6+	4+

The hydrogen potential of sperm suspensions of different concentrations at different times has been reported (Table II.). The total carbon dioxide production of each sperm suspension is equal to the difference between the carbon dioxide concentration of the sea water at the beginning of the experiment and at the time of the death of spermatozoa. If the total carbon dioxide

production is divided by the concentration of spermatozoa in the suspension, the carbon dioxide production per unit concentration of sperm is obtained.

The relative carbon dioxide production of sperm suspensions of different concentrations is reported in Table IV.

TABLE IV.

TOTAL CARBON DIOXIDE PRODUCTION OF SPERM SUSPENSIONS.

No.	Sperm Concentration.	Calculation of the Relative Carbon Dioxide Production per Unit Concentration of Sperm.	Approximate Length of Life of Spermatoza.
1	0.5%	13	17+
2	0.2%	18	17+
3	0.1%	26	17+
4	0.05%	37	10+
5	0.02%	32	6+
6	0.01%	45	6+
7	0.005%	60	4+

It is apparent in the most concentrated suspensions that sea water that is more acid than Ph 7.6 destroys spermatozoa, and causes their death before they have been able to expend all their available energy (see p. 192). A post-lethal increase in acidity occurs in such suspensions. McClendon has evidently made similar observations regarding marine invertebrates, for he says: "It would be of little advantage" to determine the carbon dioxide concentrations in sea water more acid than Ph 7.6 "unless it is first absolutely established that the abnormal Ph does not make the organisms physiologically abnormal and that oxygen is still present in the water" (McClendon, Gault, and Mulholland, 1917, p. 33).

In the less concentrated suspensions which more closely simulate normal conditions it will be seen that spermatozoa that live for longer periods of time produce no more carbon dioxide than spermatozoa that live for only 4 hours. In fact the total carbon dioxide production of spermatozoa is an approximate constant. Using the carbon dioxide production as the criterion it must be concluded that the activity of spermatozoa and therefore the life of spermatozoa is limited.

An analysis of the length of life of spermatozoa is essentially, therefore, an analysis of the rate of activity of spermatozoa under varying environmental conditions.

IV. The Effect of the Concentration of the Sperm suspension upon the Activity and upon the Longevity of Spermatozoa.

The observations and the experiments that have been cited show that sea urchin spermatozoa soon become inactive in a certain concentration of hydrogen ions or of carbon dioxide. In lower concentrations spermatozoa are the less active the higher the concentration of hydrogen ions or of carbon dioxide (see also Lillie, F. R., 1913). In the measurements of the carbon dioxide concentration of sperm suspensions that have been reported the sperm produced sufficient carbon dioxide in the more concentrated suspension to inactivate themselves in about two hours. The carbon dioxide production of inactive spermatozoa is of course very much less than the carbon dioxide production of highly active spermatozoa. Since spermatozoa are decreasingly active the higher the concentration of carbon dioxide in the suspension, the observed falling off with time of the carbon dioxide production in all of the suspensions is easily understood.

In very concentrated sperm suspensions, therefore, the spermatozoa are active for but a short time. That undiluted mammalian sperm exhibit but little activity was observed by Koelliker. He remarks: "In der Regel findet sich die Bewegung allerdings nur am Rande des Tropfens, nicht weil hier eine Verdunstung des Samens statt hat, . . . sondern weil am Rande des Tropfens die Intercellular-flüssigkeit in etwas bemerklicherer Weise sich ansammelt" (Koelliker, A., 1856, p. 205). The observation has since been confirmed by many investigators and for nearly all forms. The explanation of the activity of spermatozoa at the border of the drop is contained in the observations of Buller (Buller, A. H., 1902) and Lillie (Lillie, F. R., 1913) quoted in the last section. At the edge of a sperm suspension the oxygen concentration is higher and the carbon dioxide concentration lower than in any other part of the drop. Consequently spermatozoa accumulate in the region of highest carbon dioxide concentration. There they are very inactive, and live for a very long time.

Further evidence that the increased length of life in the more concentrated suspensions is due to the decreased production of

carbon dioxide in these suspensions is afforded by the following experiments, which are of two kinds. (1) The length of life of a sperm suspension was either increased by decreasing the rate at which the carbon dioxide produced by the sperm could diffuse from the suspension; or (2) the length of life of the sperm suspension was decreased by decreasing the rate at which the carbon dioxide and hydrogen ion concentration of the suspension increased. This again was attained in two ways. The procedures and the protocols of these experiments will now be reported.

V. The Relation between the Length of Life of a Concentrated Sperm Suspension and the Rate at which the Carbon Dioxide produced by Spermatozoa is Eliminated.

1. *Decreasing the Rate of Diffusion of Carbon Dioxide (Experiments 31, 32, and 33).*

In these experiments a sperm suspension was divided between vessels of different diameter. The area of the suspensions that was in contact with the air, and consequently the rate at which the carbon dioxide produced by the sperm escaped into the air constituted the only variable. It was found that the sperm suspensions from which the carbon dioxide could least rapidly diffuse lived for the longest time as judged by the percentage of eggs fertilized when spermatozoa from these vessels were added to eggs in sea water as a function of time (Table V.).

Two methods were employed of decreasing the rate at which the carbon dioxide produced by spermatozoa increases the carbon dioxide and hydrogen ion concentration of the suspension. The procedure in the one (*a*) was essentially the reciprocal of that employed in decreasing the rate of diffusion of carbon dioxide. In the other method (*b*) the "buffer" (Henderson, L. J., 1908) action of sea water and therefore the rate of neutralization of the carbon dioxide was artificially increased.

(2*a*) *Increasing the Rate of Diffusion of Carbon Dioxide.*

In experiment 214 the sperm suspension was divided between two shallow vessels. The one remained in contact with the air of the room, while over the surface of the other a stream of carbon

TABLE V.

THE RELATION BETWEEN THE LENGTH OF LIFE OF A CONCENTRATED SPERM SUSPENSION AND THE RATE AT WHICH THE CARBON DIOXIDE PRODUCED BY SPERMATOZOA DIFFUSES FROM THE SUSPENSION.

Experiment 32.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		A.	B.	C.	D.	No. of Drops of Sperm Added to Eggs in Sea Water.
				Approximate Area in Square Centimeters of the Interface Between Air and Sperm Suspension.				
				3.	20.	80.	500.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Four Drops Eggs in 5 c.c. of Sea Water.				
0.04%	8.10	0	0	86	62	59		1
	9.30	1	20	19	10	5		
	10.10	2	00	3	1	0		
0.04%	8.10	0	0					4
	9.30	1	20	41	13	11		
	10.10	2	00	31	3	3		
	10.25	2	15	11	0	0		
				Experiment 33.				
0.05%	12.30	0	0					1
	12.35	0	5	94		98	67	
	1.20	0	50	57		43	6	
0.05%	1.20	0	50	99		94	54	8
	2.40	2	10	69		28	7	
	3.35	3	5	34		15	4	
	4.45	4	15	16		41	3	
				Experiment 31.				
0.04%	11.07	0	0	99	100			1
	12.07	1	00	100	60			
	12.37	1	30	96	21			
	12.57	1	50	99	23			
	1.30	2	23	90	14			
	2.00	2	53	61	7			
0.04%	11.07	0	0	100	100			8
	12.07	1	00	100	100			
	12.37	1	30	100	100			
	12.57	1	50	100	64			
	1.30	2	23	100	33			
	2.00	2	53	100	35			

dioxide free air was continuously drawn. In this way the carbon dioxide concentration of the one suspension was prevented from increasing at the same rate as that of the other. As a result the

carbon dioxide production of the spermatozoa in the latter case was not so much inhibited. The life of the sperm suspension was, therefore, shortened.

TABLE VI.

THE RELATION BETWEEN THE LENGTH OF LIFE OF A CONCENTRATED SPERM SUSPENSION AND THE RATE AT WHICH THE CARBON DIOXIDE PRODUCED BY SPERMATOZOA DIFFUSES FROM THE SUSPENSION.

Experiment 214.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		*A.*	*B.*
				Approximate Carbon Dioxide Tension of the Air with which the Surface of the Suspension Was in Contact.	
				About 0.3 Mm.	About 0.1 Mm.
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop of Sperm is Added to Ten Drops Eggs in 10 c.c. of Sea Water.	
0.02%	12:00 M.	0	0		
	12.15 P.M.	0	15	100[1]	100[1]
	1.30	1	30	100[1]	100[1]
	3.30	3	30	100[1]	100[1]
	5.30	5	30	100[1]	100[1]
	7.15	7	15	100[1]	100[1]
	9.35	9	35	100[1]	85
	12.35 A.M.	13	35	100[1]	77
	1.35	13	35	100[1]	74
	10.30	22	30	90	19
	12.15 P.M.	24	15	89	20
	1.30	25	30	92	32
	5.00	29	00	93	25
	8.00	32	00	81	1
	11.50	35	50	20	0

(2b) *Increasing the Rate of Neutralization of Carbon Dioxide.*

When a mixture of a weak acid and its salt, isohydric with sea water, is added to sea water, a series of solutions is obtained that tend increasingly to maintain the reaction of the sea water. The carbon dioxide produced by spermatozoa or other organisms changes the hydrogen ion and the carbon dioxide concentration of such solutions the less, the greater the concentration of weak acids.

In the experiments to be reported mixtures of borax and boric acid were used (Palitzsch, 1914). It is, of course, necessary that neither the weak acid nor the salts of the weak acid that are

[1] Fertilization was seen to be practically complete and the percentage of eggs cleaving was only estimated.

formed with the ions of sea water be toxic to the organism under investigation. According to a personal communication, C. M. Child has observed distinct toxic action of the concentrations of borates used in these experiments upon developing sea urchin eggs. It is therefore possible that the action of borates in shortening the life of the sperm is in part due to their toxicity.

TABLE VII.

THE RELATION BETWEEN THE LENGTH OF LIFE OF A CONCENTRATED SPERM SUSPENSION AND THE RATE AT WHICH THE CARBON DIOXIDE PRODUCED BY SPERMATOZOA IS NEUTRALIZED BY ISOHYDRIC BORATES.

Experiment 208.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		*A.*	*B.*	*C.*	*D.*	*E.*	*F.*	
				No. of c.c. of Borate Mixture Added to 10 c.c. of the Sea Water in the Sperm Suspensions.						
				0.	0.1.	0.3.	0.5.	1.0.	2.0.	Ph of Borate Mixture.
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop of Sperm Added to Five Drops Eggs in 5 c.c. of Sea Water.						
0.1%	2.25 P.M.	0	0	100	100	100	100	99	100	8.41
	2.55	0	30	100	97	98	26	47	8	
	3.25	1	00	92	82	57	55	10	1	
	4.00	1	35	52	21	14	13	0	0	
Experiment 205.										
0.2%	11.20 A.M.	0	0	100	100	100	100	100		8.41
	11.50	0	30	100	100	100	99	100		
	1.40 P.M.	2	20	66	28	40	4	2		
Experiment 209.										
1.0%	10.00 A.M.	0	0	100					50	8.41
	5.20 P.M.	7	20	98	25	6	1	3	0	
Experiment 223.										
1.0%	4.00 P.M.	0	0							8.08
	10.00 A.M.	18	0	88	80	77	70			
	11.30 A.M.	19	30	85	86	27	87			
	[1] 2.00 P.M.	22	00	100	100	28	19			
1.0%	4.00 P.M.	0	0							8.51
	10.00 A.M.	18	0	88	82	3	8			
	11.30	19	30	85	80	2	2			
	2.00 P.M.	22	00	100	100	9	27			

[1] The eggs of a female that had been opened at 1.30 P.M. were used in this series of inseminations. They were evidently more easily fertilized than the eggs of the female that had been opened at 9.30 A.M.

In suspensions with the highest concentration of weak acids the spermatozoa lived for the shortest time. For the higher the concentration of weak acids the smaller the change in the hydrogen ion and carbon dioxide concentration of the suspensions due to the carbon dioxide produced by the spermatozoa. Consequently spermatozoa in more alkaline solutions, where they were most active, lived for the shortest time. In order the better to prevent spermatozoa from being inactive, a borate mixture that was slightly more alkaline than sea water was employed in the first three experiments reported (Experiments 205, 208, 209). The hydrogen potential of the borate mixture is recorded in the last column of Table VII.

That even this slight change in the hydrogen potential of the borate effects spermatozoa is demonstrated in Experiment 223 where slightly different mixtures of borates were used in the same concentration. It will be seen that spermatozoa in the suspension to which had been added the more alkaline mixture, lived the shorter time.

VI. The Effect of the Hydrogen Ion Concentration of the Suspension upon the Activity and upon the Longevity of Spermatozoa.

The activity of spermatozoa is a function of the hydrogen ion concentration. Repeated observation has confirmed this relation. Since there is a definite relation between the activity and the length of life of spermatozoa, the latter is also a function of the hydrogen ion concentration. The hydrogen ion concentration of the ocean at Woods Hole, Massachusetts, is about 0.1×10^{-7} (Ph 7.95 to Ph 8.15). The weak acids (Henderson, L. J., and Cohn, E. J., 1916) and the currents prevent the hydrogen ion concentration of the ocean from appreciably changing. In such a solution the length of life of spermatozoa is short.

Loeb has observed the simultaneous spawning of the sea urchins (*Strongylocentrotus purpuratus*) at the shore of Pacific Grove. "At such spawning seasons the sea water becomes a suspension of sperm" (Loeb, J., 1916, p. 94). It would be interesting to know whether the hydrogen ion concentration of such a suspension increases.

Such concentrations of spermatozoa as were used in the experiments reported in the last section probably never occur in the ocean. The conditions that obtained in these experiments approximate those of ripe sperm in the testes very much more closely than they do those of sperm that are shed into the ocean. The hydrogen ion concentration in these suspensions increased not inconsiderably as a result of carbon dioxide produced by the sperm. This rise in carbon dioxide (and also in hydrogen ion) concentration was measured. The hydrogen ion and carbon dioxide concentration of the suspensions in which spermatozoa lived for the longest time was sufficiently great to inactivate spermatozoa. The length of life of concentrated sperm suspensions is therefore for the most part ascribable to the hydrogen ion concentration of such suspensions.

The length of life of a sperm suspension at different hydrogen ion concentrations was determined.

Two criteria of the length of life of the sperm were employed. The fertilization tests are reported. The hydrogen potential of the sea water was determined by colorimetric comparison with standardized mixtures of borates or phosphates in the way that that has already been described (p. 171).

In the experiments to be reported the hydrogen ion concentration was decreased by the addition of sodium hydroxide to sea water. The hydrogen ion concentration was increased by the addition of hydrochloric acid.

When an acid stronger than carbonic acid is added to sea water, carbonic acid is displaced from its salts and carbon dioxide is set free. As the carbon dioxide that is set free diffuses from the solution the hydrogen ion concentration decreases, until the carbon dioxide of the sea water is again in equilibrium with the partial pressure of that gas in the air. The hydrogen ion concentration that is eventually reached is different from the original hydrogen ion concentration of the sea water, but nearer to it than to the hydrogen ion concentration immediately after the acid is added. This regulation of the neutrality persists until all of the carbonates have been decomposed. The rate at which equilibrium is approached depends upon the temperature, the surface, and the degree of agitation.

Because of this property of carbonate solutions it is necessary to know not only the hydrogen ion concentration but also the carbon dioxide tension. The latter is, as stated, above expressed by the number of millimeters of mercury that represents the partial pressure of the gas. The procedure usually followed was to restore the equilbrium between the sea water (of whatever hydrogen ion concentration) and the carbon dioxide of the air, before beginning an experiment. This was accomplished either by shaking with air, or by bubbling air through the solutions.

In a series of experiments it was found that the more alkaline the solution (*i. e.*, the lower the hydrogen ion concentration) the shorter the life of the sperm. The activity of the spermatozoa is increased in these suspensions, and spermatozoa that are added to ripe eggs in sea water while in this activated condition have a greater "fertilizing power" (this has been previously reported. See Fuchs, H. M., 1915) than spermatozoa that have been in less alkaline sea water. This lasts for a much shorter time, since the life of spermatozoa is very short in alkaline solution. (If the hydrogen potential is greater than about Ph 9.4 spermatozoa are instantly agglutinated.) In order to demonstrate the increased "fertilizing power" of spermatozoa that have been in alkaline solution it is necessary to inseminate in such dilution that the spermatozoa that have been in sea water with greater hydrogen ion concentration do not fertilize all of the eggs.

Experiment 227 illustrates both the effect of alkaline sea water in increasing the "fertilizing power" for a short time and of more acid sea water in increasing the time during which the "fertilizing power" is exhibited. The reversal in the effect of alkaline sea water upon the "fertilizing power" of spermatozoa was demonstrable only because the eggs used in the first part of this experiment were fertilized with difficulty. Otherwise the early fertilizations would have been complete, and the effect of alkaline sea water upon spermatozoa not have been observed. Diagram III. represents the prolongation of the life of the sperm in acid suspension. The ordinates measure the fertilizing power of the sperm at the times designated by the abscissæ. The hydrogen potentials of the suspensions are symbolically recorded.

If sea water is much more alkaline than Ph 9.4, spermatozoa

are instantly agglutinated. If sea water is about Ph 7.6, spermatozoa are inactive. Sea water that is much more acid than Ph 7.6 not only tends to inactivate, but also to destroy spermatozoa and the more so the higher the hydrogen ion concentration and the longer the sperm are subjected to these acidities.

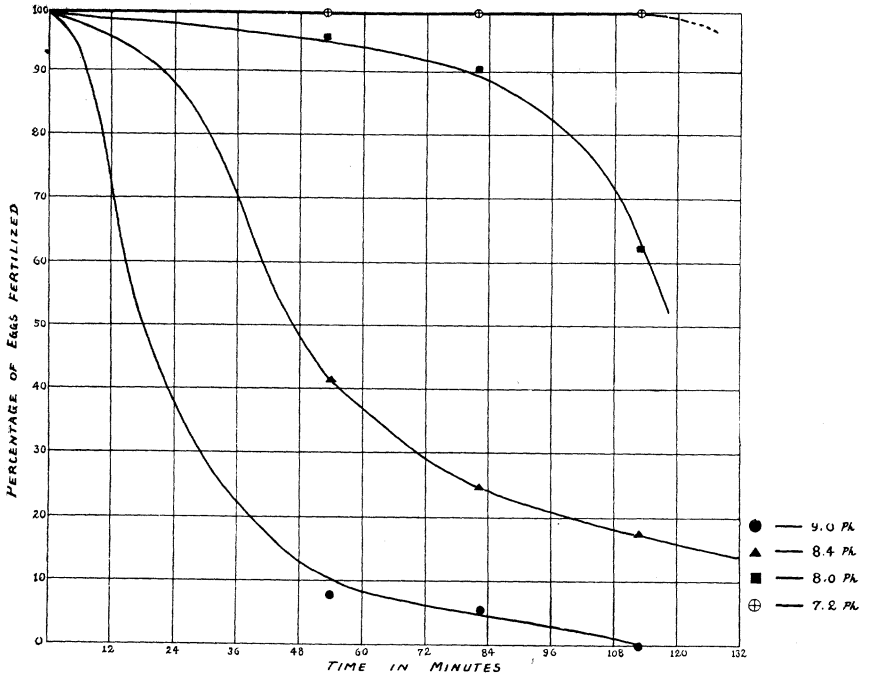

DIAGRAM III.

In sea water that is less acid than Ph 7.6 spermatozoa live the longer the higher the hydrogen ion concentration. The more inactive the spermatozoa the more slowly their "fertilizing power" diminishes when they are transferred to sea water where they are reactivated.

VII. THE RELATION BETWEEN THE ACTIVITY AND THE "FERTILIZING POWER" OF SPERMATOZOA.

"Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time" (Lillie, F. R., 1915, p. 246). The results of this investigation confirm this general conclusion of Lillie's, and

add one more factor, the hydrogen ion concentration. For the length of life and the "fertilizing power" of a sperm suspension are dependent on the hydrogen ion concentration of the suspension, and the "fertilizing power" of spermatozoa at the same hydrogen ion concentration is in some way dependent upon the sperm concentration.

The Effect of the Hydrogen Ion Concentration upon the Fertilizing Power of Spermatozoa.

The loss of "fertilizing power" of active sperm suspensions of approximately the same concentration (0.04 per cent.) at different hydrogen ion concentrations is graphically represented

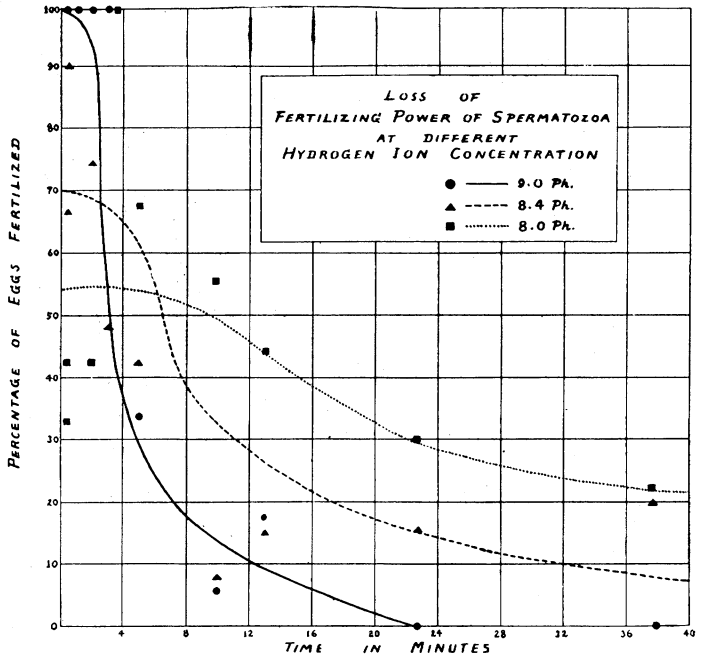

Diagram IV.

in Diagram IV. The curves are plotted from the data in Table VIII. on the basis of maximum "fertilizing power" at Ph 9.0. If but 50 per cent. of the eggs were fertilized at Ph 9.0 (as in experiment 227–I.) and 24 per cent. at Ph 8.4 the ratio of the "fertilizing powers" is of course as 100 to 48.

TABLE VIII.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION OF THE SUSPENSION UPON THE FERTILIZING POWER AND UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 20.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		C.c. of 0.02N HCl to 10 c.c. Sea Water.				C.c. of 0.2N NaOH to 10 c.c. Sea Water.				No. Drops of Sperm Added to Eggs.
				1.1.	1.0.	0.5.	0.	.06.	.12.	.25.	.50.	
				Hydrogen Potential of Sperm Suspensions at CO_2 Tension of Air.								
						7.7.	8.0.	8.2.	8.4.	8.6.	9.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm is Added to Five Drops Eggs in 5 c.c. of Sea Water.								
0.015%	11.51		0									1
	11.53		2				41	48	67	51	98	
	1.00	1	9				0	0	0	0	0	
Experiment 226.												
0.04%	4.40		0									1
	4.42		2				20	39	35	47	47	
	4.45		5				32	18	20	18	16	
	4.50		10				26	32	4	34	3	
Experiment 21.												
0.045%	12.25		0				33	94	90	90	99	4
	1.25	1	0				5	36	23	1	3	
	2.20	1	55				1	7	7	0	1	
Experiment 227—I.												
0.08%	11.07		0									1
	11.10		3	4	38	7	51		24		50[1]	
	11.20		13	11	60	15	22		8		9	
	11.30		23	19	31	18	15		8		0	
	11.45		38	13	44	0	11		10		0	
Experiment 227—II.[2]												
	12.00		53	100	100	89	96		41		8	
	12.30	1	23	100	100	67	90		25		6	
	1.00	1	53	100	100	60	63		17		0	
Experiment 22.												
0.7%	12.25		0									1
	12.40		15				100	100	100	100	100	
	1.15		50				98	100	100	100	100	
	5.20	4	55				35	86	90	5	0	

[1] But 0.3 c.c. of sodium hydroxide had been added in Expt. 227. The Ph was 8.8.

[2] Same sperm used as in 227—I. The eggs, however, of another female.

Spermatozoa that have been in alkaline sea water have a greater "fertilizing power" than spermatozoa that have been in sea water at the hydrogen ion concentration of the ocean. This becomes apparent only at dilutions such that the spermatozoa that have been in the less alkaline sea water do not fertilize all of the eggs. If all of the eggs are not fertilized, it must be either (*a*) because there are not enough spermatozoa or (*b*) because the spermatozoa are not sufficiently potent. Since the same concentration of sperm is able to fertilize completely at another hydrogen ion concentration, the first alternative does not explain the results in these experiments (Experiment 20, 21, 226, 227–I.).

If the "fertilizing power" of active spermatozoa were exactly proportional to the energy expended, the areas beneath the curves in the diagram would be exactly equal, since the total available energy of spermatozoa is practically constant (p. 183). If the relation is not as simple as this, it is at all events perfectly definite and definable, and strongly suggestive of a simple functional relation, at least during the first few minutes of the life of the sperm.[1]

The Effect of the Sperm Concentration upon the "Fertilizing Power" of Spermatozoa.

"In his epoch-making 'Expériences pour servir a l'histoire de la génération des animaux et des plantes' published in 1785 the Abbe Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog" (Lillie, F. R., 1915, p. 229). "In 1824 Prevost et Dumas confirmed these" results (Lillie, F. R., 1915, p. 229). More recently in the "Analysis of Variations in the Fertilizing Power of Sperm Suspensions of *Arbacia*" (Lillie, F. R., 1915, p. 229) that has already been quoted, F. R. Lillie demonstrated that at "a dilution of 1/10,000 per cent.," where "one can rarely find a single spermatozoön in the jelly of the fertilized

[1] Thereafter it is difficult to conceive of the physiological condition of the spermatozoön as suffering no alteration. A change in the physiological condition must in turn affect the "fertilizing power." Strong evidence for the view that the ageing of Echinid sperm affects its physiological condition has previously been presented by Dungay (1913) and Vernon (1899).

TABLE IX.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION OF THE SUSPENSION UPON THE FERTILIZING POWER AND UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 228.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		C.c. of .02*N* HCl to 10 c.c. Sea Water.					No Drops of Sperm Added to Eggs.
				1.0.	0.9.	0.8.	0.5.	0.0.	
				Hydrogen Potential at CO_2 Tension of Air.					
						7.4.	7.7.	8.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm is Added to Five Drops Eggs in 5 c.c. of Sea Water.					
0.001%	11.55		0						1
	12.00		5		6	12	31	19	
	12.15		20		1	0	4	2	
	1.00		65		0	0	0	0	
0.005%	11.55		0						1
	12.00		5		41	71	72	86	
	12.15		20		1	0	3	29	
	1.00		65		0	0	1	0	
0.01%	11.55		0						1
	12.00		5		71?	31	94	100	
	12.15		20		7	1	78	86	
	1.00		65		0	0	33	5	
				Experiment 215.					
0.25%	12.05		0						1
	12.30		25	84		78	86	84	
	9.00	8	55	12		66	87	6	
	12.40	12	35	0		7	23	0	

eggs" (Lillie, F. R., 1915, p. 234) about 95 per cent. of the eggs were fertilized if the time, which "is an extremely important factor with reference to fertilizing power" (Lillie, F. R., 1915, p. 234) during which the sperm were in sea water, was short. For: "If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration" (Lillie, F. R., 1915, p. 239).

I believe that Lillie's results have conclusively demonstrated that one sperm is quite sufficient to fertilize an egg if it has not already expended a large part of its available energy. Thereafter the statement that "the initiation of development by a single spermatozoön . . . is impossible because a single sperm

cannot affect those changes in the egg-coverings" (Glaser, O., 1915, p. 153) which are necessary to fertilization, has unquestioned significance. The older the spermatozoa and the more their available energy has been expended, the more spermatozoa are necessary in order to effect fertilization. There is, however, even then no doubt that "a single spermatozoön is sufficient to carry out the bi-parental effect" (Glaser, O., 1915, p. 153).

The "fertilizing power" of sperm at the same hydrogen ion concentration is, therefore, in some way dependent on the concentration of the sperm. In Experiment 228 in Table IX. (p. 196) a 0.001 per cent.; a 0.005 per cent. and a 0.01 per cent. suspension was made from the sperm of one male. The "fertilizing power" at each hydrogen ion concentration was lost the sooner the smaller the concentration of sperm.

It is, of course, true that in all but the most dilute suspensions the hydrogen ion concentration will in a short space of time be the higher the more concentrated the suspension as a result of the carbon dioxide produced by the sperm. This probably occurred in Experiment 22, but it is improbable that this was the only factor in the other experiments, and it certainly was not a factor in the data that will now be presented.

In Experiments 31, 32 and 33 (p. 186) different amounts of sperm from the same suspension were added to the same concentration of eggs. These experiments are retabulated in order that the percentage of eggs that were fertilized by different amounts of sperm from the same suspension at the same time can more easily be compared. When all of the eggs were not fertilized by both concentrations of sperm the number of eggs fertilized was always the greater the more spermatozoa were added.[1]

This is not a new observation. It is the common experience of investigators, and may be explained either by assuming that in the greater concentration more sperm will on the average arrive at the periphery of the egg with sufficient action (having the dimensions of energy $\times$ time) to initiate development, or by assuming that mass action of spermatozoa may be effective in

[1] It should be pointed out that whereas motility is unquestionably a manifestation of energy, the observation of motile spermatozoa at the periphery of an unfertilized egg in no way indicates the physiological condition of the spermatozoa.

initiating the development of the egg. This implies that what is true in parthogenesis is also true in normal fertilization. R. S. Lillie (Lillie, R. S., 1916) has quite beautifully shown that the product of the concentration of the parthenogenetic agent and the length of time during which it is necessary to subject eggs to the agent in order to initiate development is a constant. The evidence that has been presented strongly suggests a similar quantitative relation in fertilization.

TABLE X.

THE RELATION BETWEEN THE CONCENTRATION OF THE SPERM AND THE PERCENTAGE OF EGGS THAT ARE FERTILIZED.

Number of Experiment.	Designation of Suspension.	Age of Sperm Suspension.		Amount of Sperm Added.		Amount of Sperm Added.	
		Hrs.	Min.	1 Drop.	4 Drops.	1 Drop.	8 Drops.
				Percentage of Eggs that are Fertilized.		Percentage of Eggs that are Fertilized.	
32	*A*	1	20	19	41		
	B	1	20	10	13		
	C	1	20	5	11		
	A	2	0	3	31		
	B	2	0	1	3		
	C	2	0	0	3		
33	*A*		50			57	99
	B		50			43	94
	D		50			6	54
31	*A*	1	0			100	100
	B	1	0			60	100
	A	1	30			96	100
	B	1	30			21	100
	A	1	50			99	100
	B	1	50			23	64
	A	2	23			90	100
	B	2	23			14	33
	A	2	53			61	100
	B	2	53			7	35

VIII. THE EFFECT OF THE CARBON DIOXIDE CONCENTRATION UPON THE ACTIVITY AND UPON THE LONGEVITY OF SPERMATOZOA.

The effect of increasing the carbon dioxide concentration of the suspension is to increase the length of life of the spermatozoa.

TABLE XI.

THE EFFECT OF THE CARBON DIOXIDE TENSION UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 225.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		CO_2 Tensions of the Suspensions in Mm.					No. Drops Sperm Added to Eggs.
				16.	4.	2.	1.	0.3+.	
				Hydrogen Potentials of the Suspensions.					
				6.5.	7.0.	7.3.	7.6.	8.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Eggs in 5 C.C. of Sea Water.					
0.04%	4.40	0	0						1
	9.00	4	20	10	7		97	53	
	11.45	7	5	0	4		41	16	

Experiment 212.

0.2%	12.05	0	0						1
	12.10		5	100	100		100	100	
	12.25		20	100	100		100	100	
	2.30	2	25	100	100		100	100	
	4.40	4	35	99	100		100	100	
	11.50	11	45	100	100		100	100	
	9.55	21	50	7	4		94	80	
	12.26	24	15	1	0		100	100	
	4.50	28	45	2	15		92	90	

Experiment 213.

0.2%	12.10	0	0						1
	12.20	0	10			100		100	
	1.50	1	40			100		55	
	3.30	3	20			98		96	
	4.50	4	40			100		99	
	9.40	9	30			99		95	
	9.40	21	30			100		20	
	12.15	24	5			84		5	

For as the carbon dioxide concentration of sea water increases, so also does the concentration of carbonic acid and of hydrogen ions. In several experiments the length of life of a sperm suspension at different carbon dioxide tensions[2] was determined and sperm were found to live for the longest time when the carbon dioxide tension was about one millimeter. The hydrogen potential of sea water at a carbon dioxide tension of one milli-

[1] In this experiment five drops of eggs were added to the undiluted suspensions.

[2] The relation between the tension and the concentration of carbon dioxide is discussed on page 172.

meter is about 7.6 (Henderson, L. J., and Cohn, E. J., 1916). But this is the hydrogen potential that has been found to be most effective in increasing the length of life of spermatozoa. Three experiments are reported in Table XI. It will be seen that when the carbon dioxide tension is greater than one millimeter and the hydrogen potential less than 7.6, sperm are destroyed. This is in complete agreement with what has already been found with respect to the hydrogen ion concentration.

It is not maintained that the only effect of carbon dioxide upon the physiological condition of spermatozoa is brought about by ionized hydrogen. On the contrary, several experiments, which are unfortunately not conclusive (and are therefore not reported) indicate a difference in the subsequent behavior of spermatozoa that are subjected to the same hydrogen ion concentration but to different carbon dioxide tensions. The fact that the total carbon dioxide production per unit concentration of sperm in concentrated suspensions is less than in more dilute suspensions (Table IV.) suggests that the rate at which carbon dioxide is eliminated depends in some way upon the difference in carbon dioxide tension between the spermatozoon and its environment.

IX. The Effect of the Oxygen Concentration upon the Activity and upon the Longevity of Spermatozoa.

The generalization may be hazarded that whatever decreases the activity increases the length of life of spermatozoa and conversely that whatever increases the activity decreases the length of their life. Buller (Buller, A. H., 1902) observed the differential activity of spermatozoa in an oxygen gradient (see p. 175). Drzewina and Bohn (Drzewina, A., and Bohn, G., 1912) have demonstrated that sperm live for a long time in an oxygen-poor medium.

Drzewina and Bohn have also demonstrated that the addition of KCN to sea water prolongs the life of the sperm. Loeb (1915) has shown that spermatozoa are immobilized by NaCN, and it is certain that the length of their life is thereby increased. "It is a well-known fact that the unfertilized eggs of the sea urchin (in fact of all marine animals) perish when they lie for some time in

sea water and one of the main causes of this phenomenon is also known, namely oxidations. If the oxidations are inhibited through the removal of oxygen or the addition of KCN the life of the eggs can be prolonged"[1] (Loeb, J., 1915, p. 282).

It is probable that oxygen lack also plays a part in increasing the length of life of concentrated sperm suspensions. McClendon (McClendon, Gault and Mulholland, 1917) has recently estimated that respiration that raises the hydrogen potential of sea water (of excess base 23) to approximately 7.6 uses up all of the available oxygen.

The experiments of Drzewina and Bohn have been repeated and the results substantially confirmed. Spermatozoa are quite inactive in the concentrations of KCN that are most effective in prolonging the life of spermatozoa. The results of several experiments follow.

TABLE XII.

THE EFFECT OF THE ADDITION OF KCN TO SEA WATER ON THE LENGTH OF LIFE OF THE SPERM SUSPENSION.

Experiment 14.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		Number of C.C. of 0.1*N* KCN Added to a Liter of Sea Water.								No. of Drops Sperm Added to Eggs.
				10	2.5	1.25	0.62	0.31	0.16	0.04	0	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Eggs in 5 C.C. Sea Water.								
0.04%	11.05	0	0	99	97		97		93	97	71	8
	12.05	1	0	100	98						91	
	1.40	2	35	1	100						34	
	2.45	3	40	2	100		79		16	26	4	
	7.15	8	10		73							

Experiment 27.

0.7%	3.45	0	0									1[2]
	4.40	0	55		100	100	100	100			100	
	9.50	18	5		100	100	100	100			100	
	9.45	42	0		100	5	99	98			97	
	2.00	46	15		98	0	4	2			2	
	4.45	49	0		97		6	0			0	
	7.00	51	15		2		1					

[1] The way in which KCN effects the oxidations of cells need not be discussed in connection with these experiments. For a discussion of this question see Lillie, R. S., 1916, page 311, and Child, C. M., 1915, p. 66).

[2] Insemination took place in 10 c.c. of sea water in this experiment.

X. The Effect of Beef Broth upon the Activity and upon the Longevity of Spermatozoa.

Gemmill showed that the length of life of spermatozoa could be increased by adding beef broth to sea water. He believed that this brought about the "keeping alive of spermatozoa by artificial nutrition" (Gemmill, J. F., 1900, p. 171) (see p. 178).

The work of Gemmill has been repeated and his observation that sea water to which beef broth has been added in appropriate concentration prolongs the life of spermatozoa, confirmed. The activity of spermatozoa, however, is decreased in the beef broth suspensions. Moreover the addition of beef broth to sea water increases the hydrogen ion concentration.[1]

It is unfortunately not possible to neutralize the suspension without throwing down a heavy precipitate.[2] This brings about such changes in the ionic composition of the sea water as to make uninterpretable the results of experiments carried out in such a medium.

The protocols of two experiments that confirm the results of Gemmill are recorded.

2.5 grams of Armour's "extract of beef" were dissolved in 290 c.c. of sea water, making the concentration by weight approximately 8.6 per cent. The suspension of beef broth in sea water was acid to neutral red. That is, the hydrogen ion concentration was greater than Ph 6.5. Diluting the suspension at once decreased its concentration in beef broth and in hydrogen ions. The hydrogen ion concentrations were not directly determined, but their approximate value has been calculated from the number of cubic centimeters of 0.01*N* NaOH required to make them alkaline to phenolphthalein.

To 10 c.c. of the different concentrations of acid broth one drop of dry sperm was added. Tests were made by adding one drop of each sperm suspension to five drops of eggs in 10 c.c. of sea water. The percentage of eggs that were fertilized are reported in Table XIII.

[1] The increase in viscosity of the suspension, and also the increase in protein content, may be effective in decreasing the activity of spermatozoa.

[2] In all probability an insoluble calcium salt or aggregate.

TABLE XIII.

THE EFFECT OF THE ADDITION OF BEEF BROTH TO SEA WATER UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 35.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		Concentration of Beef Broth in Sea Water. 8.6%.	4.3%.	2.1%.	1.1%.	0.5%.	0.0%.	No. of Drops Sperm Added to Eggs.
				Probable Hydrogen Potential of the Suspensions. 6.5.	7.2.	7.6.	7.8.	7.9.	8.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Five Drops Eggs in 10 C.C. of Sea Water.						
0.02%	11.55	0	0	61	81	99	99		93	1
	12.25	0	30	2	92	100	58		0	
	12.48	0	53	0	61	88	99		2	
	1.15	1	20	0	5	79	67		0	
	1.45	1	50	0	0	2	46		0	
				Experiment 34.						
0.7%	10.05	18	35	0	98	99	98	99	97	1
	2.45	23	15	0	94	97	95	98	96	
	6.20	26	50	0	53	95	94	79	30	
	10.10	30	40	0	32	89	98	43	20	

XI. THE EFFECT OF "EGG WATER" UPON THE ACTIVITY AND UPON THE LONGEVITY OF SPERMATOZOA.

Owing to the fact that sperm are "agglutinated" by the water in which eggs of the same or closely related species have been allowed to stand, much interest has centered around the effects of the so called "egg water" or "fertilizin" upon the fertilizing power of spermatozoa.

A. H. Buller seems to have been one of the first students of the fertilization process in Echinids to have noticed this phenomenon. In 1900 he reported before the British Association: "In the case of *Arbacia* it was discovered that when spermatozoa are introduced into a drop containing freshly extruded eggs they collect into small balls, often composed of 100 or more individuals. The balls were also formed after the water had received four successive filtrations. A tactile stimulus appears to play a part in the phenomenon" (Buller, A. H., 1900, p. 387).

Since then this phenomenon and the properties of the agglu-

tinating "egg water"[1] have been successively studied by E. von Dungern (1901) and (1902); A. Schücking (1903) J. De Meyer (1911); F. R. Lillie (1912, 1913, 1914, 1915); H. M. Fuchs (1915); Jacques Loeb (1914, 1915); Otto Glaser (1913, 1914); A. Richards and A. E. Woodward (1915) and A. E. Woodward (1915). Each investigator has conceived the function of the "egg water" and its importance in the fertilization process to be different. It is not the purpose of the present communication to consider the function of the "egg water" (although that is a problem of great biological interest) but the behavior of the spermatozoön, and it has been possible to repeat and to explain many of the seemingly contradictory observations of different investigators on the effect of "egg water" upon the fertilizing power and upon the length of life of spermatozoa.

The effect of "egg water" upon spermatozoa as was clearly shown in the admirable investigation of Schücking depends upon the relative concentration of egg water and sperm; upon the absolute concentration of each; and upon the length of time during which sperm are allowed to remain in the egg water. Schücking observed that: "Die sauer reagirende Eimasse übt bei den genannten Echinodermen eine tödtliche, bei kurzer Dauer der Einwirkung lähmende, in geringer Menge agglutinirende bezw. erregende und anlockende Wirkung auf Spermien der eigenen und fremden Art aus" (Schücking, A., 1903, p. 91).

In a more complete analysis of the phenomenon of activation and agglutination F. R. Lillie (1913) showed that if "egg water" is added to a sperm suspension the activity of the spermatozoa is greatly increased. One of the manifestations of this increased activity is the "agglutination" phenomenon. According to Gray "if a drop or two of a very weak solution of cerous chloride is added to a suspension of *Arbacia* sperm in sea water the spermatozoa become intensely active, and rapidly aggregate into clumps" (Gray, J., 1915, p. 123). This may possibly be (Lillie, F. R., 1915, p. 20) what Lillie now calls "mass coagulation," which was described by Loeb in 1904 (Loeb, J., 1904) and is favored not only by increase in the hydroxyl ion concentration

[1] The distilled water "extract" of Echinid eggs has been found to possess many of the properties of the "egg water."

but by increase in the concentration of bivalent ions, notably calcium (Loeb, J., 1914). Unlike "agglutination" it is not reversible. In an earlier paper Lillie did not distinguish between the two phenomenon, for he wrote: "Agglutination is not in itself a specific process; it may take place spontaneously to a certain extent under some conditions; it is caused by increase of alkalinity of the sea-water" (Lillie, F. R., 1913, p. 563). Loeb has designated Lillie's "agglutination" as "cluster formation."

It will be remembered that increasing the alkalinity also increases the activity of spermatozoa. Loeb (1914) has shown that inactive sperm do not exhibit the reversible "agglutination."[1] The irreversible agglutination, or "mass coagulation" is independent of the motility of the spermatozoa.

If the "egg water" is of sufficient strength,[2] however, the sperm are completely non-motile after the initial period of activation. By adding eggs to such spermatozoa Lillie showed that their fertilizing power was slight. "The powerful effect of the egg extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power" (Lillie, F. R., 1913, p. 558).

Fuchs (Fuchs, H. M., 1915) in experiments in which sperm that had been treated with "egg water" were added to eggs in sea-water showed that the fertilizing power of the sperm had been increased by the "egg water."[3]

But an analysis of the effect of "egg water" upon the fertilizing power of spermatozoa must differentiate between the following

[1] That "agglutination" is reversible may possibly be attributable to an increase in the acidity of the clusters; the result of the carbon dioxide produced by the tremendously active spermatozoa. In alkaline medium the carbon dioxide would be neutralized.

[2] In the measurement of the strength of "egg water" the method of F. R. Lillie, (namely considering that dilution of "egg water" that gives a visible "agglutination" as unity), is adopted. Reference is made to the papers of Lillie, F. R., and Fuchs, H. M., for a detailed description of the methods and precautions employed in this type of experimentation.

[3] According to T. B. Robertson, "when spermatozoa are washed in 3/8 *M* $SrCl_2$ and then in blood serum, they gain an added potency in fertilizing." (Robertson, T. B., 1913, p. 128.) The same treatment agglutinates (*ibid.*, page 71) (and also cytolyzes) (*ibid.*, p. 91) the eggs of the sea urchin.

effects of "egg water" upon the physiological condition of spermatozoa.

When sperm are added to egg water, their activity is tremendously increased. If they are then immediately transferred to the ripe eggs of the same species in sea water it is to be supposed that their fertilizing power will for a very short time be at least as great, if not greater than the fertilizing power of less active sperm. The experiments by Fuchs seem to have been conducted in this way. The experiments which are recorded in Table XIV. were carried out in such a way as to make the time during which the sperm were in the "egg water" as short, and the volume of sea water in which the sperm were added to eggs as great, as possible. A few experiments appear to agree with those of Fuchs in that the "egg water" increased the fertilizing power of the sperm. The results are irregular, however, for if the concentration of the "egg water" is too great, or if the time during which spermatozoa are in the "egg water" is too long, so that the activity of the spermatozoa is decreased, the fertilizing power of spermatozoa is not so great as is that of spermatozoa that have been in sea water. This seems to have been the case in the experiment of Lillie quoted by Fuchs; in which "to five watch glasses containing each eight drops of water or of different concentrations of egg-extract were added three drops of 'opalescent' sperm-suspension. After 12 minutes, a drop of a suspension of fresh eggs was added to each.' 5 per cent. of the eggs in the water segmented, but none of those in the four different concentrations of extract" (Fuchs, H. M., 1915, p. 275; Lillie, F. R., 1913, p. 558). It will be noted that in this experiment of Lillie's insemination took place in the egg extracts. Repeating this procedure as nearly as possible, Fuchs was able to obtain higher percentages of fertilization in his "extracts" than in the sea water. This difference in the effect of egg "extracts" upon the fertilizing power of spermatozoa is probably due to the relative concentration of the "extract" and the sperm, and to the absolute strength of the former. For, as Schücking early observed, although spermatozoa are stimulated by low concentrations they are "lamed" (that is, their activity is temporarily decreased) by

TABLE XIV.

THE EFFECT OF "EGG WATER" UPON THE FERTILIZING POWER OF SPERMATOZOA.

Experiment 8.[1]

Concentration of Suspension.	Concentration at Insemination.		Age of Sperm.		Approximate Concentration of "Egg Water."							
					640.	128.	64.	16.	8.	4.	1.	0.
	Drops of Sperm.	Vol. of Sea Water.	Hrs.	Min.	Percentage of Eggs Fertilized by Sperm.							
0.04%	1	50 c.c.	0	0				44		59		12
			0	5				17		9		14
	1	200 c.c.	0	0				18		52		23
			0	5				36		11		9

Experiment 12.

0.04%	1	50 c.c.	0	2			98		87		86	78
			0	5			41		76		58	37
			0	10			75		34		10	77
	8	50 c.c.	3	10			22		18		25	14

Experiment 11.[2]

0.04%	1	50 c.c.	0	0		19				11		16
			0	5		15				21		10
			0	10		11				38		

Experiment 16.

0.013%	1	5 c.c.	0	3	48		53	52		42	45	27
											81	
			1	22	43		62	10		8	6	11

Experiment 5.[3]

0.04%	8	5 c.c.	0	5				49		36	28	19
			0	10				21		26	6	2
			1	00				37		27	8	4
			2	00				42		8	3	2
			5	15				0		0	0	0

Experiment 10.

0.04%	1	50 c.c.	0	3			68	75		95	94	100
			0	8			89	10		83	21	94
			3	50			87	10		61	67	62

[1] In Experiment 8 the strength of the "egg waters" was as 3 to 1, but the "egg water" gave a 60-second reaction.

[2] In Experiment 11 the strength of the "egg waters" was as 125 to 5.

[3] In Experiment 5 the strength of the "egg waters" was as 16:4:1.

higher concentrations of egg "extract."[1] In certain of the experiments that are recorded the "fertilizing power" of the sperm was increased in certain concentration of "egg water" but decreased in greater concentration.

TABLE XV.

THE EFFECT OF "EGG WATER" UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 17.[2]

Concentration of Suspension.	Concentration at Insemination.		Age of Sperm.		Approximate Concentration of "Egg Water."									
					1280.	640.	128.	64.	32.	16.	8.	?.	2.	0.
	Drops of Sperm.	Vol. of Sea Water.	Hrs.	Min.	Percentage of Eggs Fertilized by Sperm.									
0.013%	2	5 c.c.	1	0		38	57	76						7

Experiment 13.[3]

0.04%	8	5 c.c.	0	2	100		100			100			100	100
			1	0	100		100			99			96	91
			2	2	98		91			64			42	60
			3	40	99		82			60			26	30
			5	0	95		40			7			8	10
			7	0	54		8			1			2	2

Experiment 19.[4]

1.0%	1	6.7 c.c.	19	40		66	97			99				0
			24	35		16	77			100				0
			43	40		0	0			0				0

Where the relative concentration of the "egg water" and sperm is such that the activity of the sperm is decreased, the length of life of the sperm, as measured by their ability subsequently to fertilize the ripe eggs of the same species in sea water, is greater than is that of sperm that exhibit constant activity in sea water for an equal length of time. This is to be expected since the activity of spermatozoa is limited (Cohn, E. J., 1917). Whereas the fertilizing power of sperm that have been in "egg water" of various concentrations for but a few moments is often

[1] All of the properties of "egg water" and "egg extract" are not the same. Sperm are, however, activated and agglutinated by both.

[2] The actual strength of the "egg water" used in Experiment 17 is not known. In all three suspensions, however, it gave at least a three minute agglutination.

[3] An egg extract was used in Experiment 13.

[4] An egg extract was used in Experiment 19. The strengths of the egg extracts were as 1:5:25. The weakest gave a 70-second agglutination.

smaller than that of sperm that have been in sea water, the fertilizing power of the latter is soonest lost. Schücking reports an experiment of this kind. "Wenn einer grösseren Spermienmenge ein geringes Quantum Eisubstanz zugesetzt war, so dass die Samenfäden nur gelähmt wurden, so konnten die Spermien noch nach 12 Stunden durch Zusatz von Seewasser wieder beweglich und befruchtungsfähig gemacht werden, während die in Seewasser gebrachten Spermien je nach der Temperatur nach fünf bis acht Stunden abgestorben waren" (Schücking, A., 1903, p. 59).

Since the length of time that "egg water" preserves the life of sperm depends upon the relative concentration of the "egg water" and of the sperm, and since the ability of "egg water" to preserve the life of the sperm depends on the decreased activity of the sperm that follows the initial activation, the sooner the sperm become non-motile (or exhibit decreased activity) the longer the span of their life. It is therefore obvious that a very concentrated "egg water" will often be less effective in preserving their life than a less concentrated "egg water." In Table XV. such conditions evidently obtained. Distilled water extracts of eggs (made isotonic by the addition of sea water that had been concentrated by evaporation) such as were employed by Schücking were used in Experiments 13 and 19. Egg extracts can be obtained with very great "agglutinating strength."

In the experiments reported in Table XIV. the sperm suspensions were, for the most part, made in weaker egg waters. (The egg waters employed in different experiments were probably not of exactly equivalent concentration. The concentrations reported are only approximate.) Moreover sperm was added to eggs in large volumes of sea water. Under such conditions the length of life of spermatozoa, as measured by the loss of fertilizing power, is relatively short.

A large part of the effect of "egg water" in preserving the life of spermatozoa is attributable to the hydrogen ion concentration of these solutions.[1] Lillie, F. R., remarked: "That the sea water

[1] Schücking, A., also ascribed this property of egg "extracts" to their acidity. The acidity in the case of his distilled water "extract" of Echinid eggs he believed to be due to mono-sodium and mono-calcium phosphate. The acidity in these experiments was due to the carbon dioxide production of the eggs, for the "egg water" of eggs that had stood in sea water for but short periods of time was used.

which has stood over eggs combines both the effects of an acid, (aggregation) and also an alkali (agglutination) on the spermatozoa" (Lillie, F. R., 1913, p. 549). The acidity is due to the carbon dioxide that the eggs give forth into the sea water together with any other substances that they may secrete. This carbon dioxide may be removed, and the hydrogen ion concentration of the "egg water" decreased. In that case the ability of the "egg water" to preserve the life of the sperm is much decreased, but is still greater than is that of sea water of exactly the same hydrogen ion concentration. An experiment comparing the effects of egg waters and of sea waters of different hydrogen ion concentrations illustrates this relationship.

TABLE XVI.

THE EFFECT OF "EGG WATER" AT DIFFERENT HYDROGEN ION CONCENTRATIONS UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 217.[1]

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		Egg Water.		Sea Water.	
				CO_2 Tension.		CO_2 Tension.	
				1.1.	0.8.	1.1.	0.3.
				Hydrogen Potential.		Hydrogen Potential	
				7.5.	7.7.	7.5.	8.0.
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop Sperm Added to Five Drops Eggs in 5 C.c. Sea Water.			
0.1%	2.45	0	0				
	3.00	0	15	100	100	100	98
	5.00	2	15	100	97	85	17
	6.00	3	15	100	96	70	8
	7.00	4	15	100	65	15	0
	8.00	5	15	100	47	14	0
	9.15	6	30	91	13	1	0

That the ability of "egg water" to preserve the life of spermatozoa is greater than is that of sea water of exactly the same hydrogen ion concentration is probably in large measure due to

[1] Preparation of egg water: 3 c.c. of eggs that had been strained into sea water at 10 A.M. were allowed to stand in 75 c.c. of sea water until 12.30 P.M. 70 c.c. of the supernatent fluid were then decanted and divided into two 35 c.c. solutions. The hydrogen potential was 7.5, which corresponds to a CO_2 tension of 1.14 mm. The CO_2 tension of the one solution was slightly decreased by passing room air through it until the hydrogen potential was about 7.65 and the CO_2 tension 0.85 mm.

the higher carbon dioxide and hydrogen ion concentration that is soon reached as a result of the tremendous activity of spermatozoa in "egg water."[1]

After removal of the carbon dioxide, "egg water" can still agglutinate sperm.[2]

Schücking also found that: "Zwei Tropfen des nicht dialysirten sauren Extracts oder ein bis zwei Tropfen des Rückstandes vom Dialysat, mit einem Tropfen 3 per cent. iger Na_2CO_3 Lösung und fünf Tropfen destillirten Wassers agglutinirten das Sperma noch, obgleich sie neutrale Reaction zeigten" (Schücking, A., 1903, p. 61). Accordingly spermatozoa that are added to egg water, where they are very active, produce a large amount of carbon dioxide. This is also true when spermatozoa are put into alkaline sea water. Unlike the former case, however, the carbon dioxide produced is not so effectively neutralized in the case of egg water, and the acidity of the solution rapidly increases. This is followed, as has been shown, by a decreased activity on the part of spermatozoa and a consequent increase in the length of their life.

The effect of egg water upon the fertilizing power of spermatozoa, is, therefore, not so very dissimilar to the effect of the other

[1] The writer is aware of several other possible interpretations of the observation, that (*a*) the ability of "egg water" to preserve the life of the sperm is greater than is sea water of exactly the same hydrogen ion concentration and that (*b*) a very concentrated "egg water" will often be less effective in preserving life than a less concentrated "egg water." For if the "agglutinating substance" be protein in character, as the investigation of Schücking (Schücking, A., 1903) (who found the "agglutinating substance" in the undializable fraction of an egg extract) and of Richards & Woodward (Richards, A., and Woodward, A. E., 1915) suggests the "Studies in the Fertilization of the Eggs of a Sea Urchin (*Strongylocentrotus purpuratus*) by Blood-Sera, Sperm, Sperm-Extract, and other Fertilizing Agents" of T. B. Robertson (1912) may possibly explain these observations. He observed that the "potency of the serum" in initiating either the cytolysis or the development of the egg "obtains a maximum at a dilution of about 1/16" and that "serum of this dilution frequently agglutinates the eggs, causing them to aggregate in large clumps within a few seconds" (Robertson, T. B., 1912, p. 71). In higher concentration the proteins inhibit the imbibition of water by the eggs.

[2] That the "agglutination" phenomenon is not independent of the hydrogen ion concentration of the solution has been shown by Loeb. In acid solution where the sperm are inactive, the "agglutination" cannot occur. The "agglutination" is strongest in solutions slightly more acid than is ordinary sea water but "the clusters" disappear the more rapidly the more alkaline the solution.

substances that have been studied, and can best be understood by analyzing the effect of egg water upon the physiological condition of spermatozoa. That spermatozoa are activated to an exceptional degree by a secretion from the egg has clearly been demonstrated by the work of all of the investigators who have been quoted. It may be that the fertilizing power of spermatozoa that are added to eggs in sea water during this period of activation is increased in much the same way that the fertilizing power of spermatozoa is increased for a short period of time by decreasing the hydrogen ion concentration (increasing the alkalinity) of sea water. If the sperm are added to eggs after they have become inactive, the percentage of eggs that are fertilized depends upon the degree of inactivity and the degree of reactivation (such as is brought about by a transfer to sea water without a high carbon dioxide tension) which the experimental conditions afford. The sooner the sperm become inactive and the more completely inactive they become the longer their life. Thus, excepting for the initial period of activation, the effect of "egg water" upon the length of life of the spermatozoön is essentially that of a solution of optimal hydrogen ion concentration. The hydrogen ion concentration of the "egg waters" that is most effective in preserving the life of the sperm is precisely that of the acidified sea water that best subserves the same function. In the "egg water" this hydrogen ion concentration is reached as a result either of the carbon dioxide produced by eggs and eliminated into the "egg water" or that produced by spermatozoa during the period of their greatest activity.

XII. Summary.

1. The total available energy of spermatozoa, as measured by total carbon dioxide production, is a constant.

2. The rate at which the total available energy is expended is a function of the activity and an inverse function of the length of life of spermatozoa.

3. The activity and the length of life of spermatozoa is a function of the temperature, and of the hydrogen ion concentration of the suspension. Up to a certain point decreasing the temperature or increasing the hydrogen ion concentration decreases the

activity and increases the length of life of spermatozoa. Lower temperatures or greater acidities destroy spermatozoa, and the more so the longer they are subjected to these abnormal media. Conversely, within limits, decreasing the hydrogen ion concentration or increasing the temperature increases the activity and decreases the length of life of spermatozoa. Further increase in temperature or in alkalinity irreversibly agglutinates spermatozoa.

4. The ability of spermatozoa to fertilize eggs of the same species is a function of their activity (as measured by their carbon dioxide production, or its converse, their length of life). Spermatozoa lose their power to fertilize as a function of the time of insemination, and of the dilution and of the hydrogen ion concentration of the suspension. If spermatozoa that have been in the suspension for but a short time are added to eggs in a large volume of sea water a decrease in the hydrogen ion concentration of the sperm suspension (that is, an increase in the alkalinity) increases the activity and the fertilizing power of the spermatozoa. Such spermatozoa will, however, lose their power to fertilize when transferred to ripe eggs in sea water long before spermatozoa that have been relatively inactive in more acid sperm suspensions.

5. If spermatozoa that have been at the same hydrogen ion concentration are added to eggs in sea water at the same time, increasing the concentration of spermatozoa increases the percentage of eggs that are fertilized. This may be explained either by assuming that in the greater concentration more spermatozoa will on the average arrive at the periphery of the egg with sufficient action (having the dimensions of energy $\times$ time) to initiate development, or by assuming that mass action of spermatozoa may be effective in initiating the development of the sea urchin egg. The latter explanation has been suggested by Glaser, who pointed out that the further assumption that more than one spermatozoön was necessary "to carry out the bi-parental effect" was not involved.

6. Increasing the carbon dioxide concentration increases the hydrogen ion concentration of the suspension, decreases the activity of spermatozoa and increases the length of their life.

Sea water of the optimum carbon dioxide concentration for increasing the length of life of spermatozoa is also of the optimum hydrogen ion concentration. It is possible, however, that carbon dioxide affects the physiological condition of spermatozoa otherwise than by means of ionized hydrogen.

7. That decreasing the oxygen concentration of sea water or decreasing the oxidations of spermatozoa by adding KCN to sea water increases the length of their life has been reported by Drzewina and Bohn. Experiments confirming their results and demonstrating that under such conditions sperm are relatively inactive are reported.

8. Spermatozoa increase the carbon dioxide and, therefore, the hydrogen ion concentration of concentrated sperm suspensions to the optimum for decreasing their own activity in a very short time. In such suspensions sperm live for a very long time. McClendon has recently estimated that respiration in sea water that raises the carbon dioxide and the hydrogen ion to a concentration approximately equal to that which is most effective in prolonging the life of a sperm suspension, uses up all of the available oxygen. It is therefore suggested that high carbon dioxide and hydrogen ion concentration and low oxygen concentration are the environmental conditions that obtain in a concentrated sperm suspension. These conditions approximate those of ripe sperm in the testes very much more closely than they do those of sperm that are shed in the ocean.

9. The addition of beef broth to sea water increases the length of life of the spermatozoa. Gemmill suggested that beef broth furnished "artificial nutrition." It is pointed out that the addition of beef broth to sea water increases the hydrogen ion concentration and decreases the activity of spermatozoa.

10. Sea water that has contained the eggs of the sea urchin, *Arbacia*, tremendously activates spermatozoa. It may be that the fertilizing power of spermatozoa that are added to eggs in sea water during this period of activation is increased in much the same way that the fertilizing power of spermatozoa is increased for a short period of time by decreasing the hydrogen ion concentration (increasing the alkalinity) of sea water.

Subsequent to the period of activation spermatozoa become

inactive in egg water. Such egg water has a high hydrogen ion concentration. This is reached as a result either of the carbon dioxide produced by eggs and eliminated into the sea water, or by spermatozoa during the period of their greatest activity. If spermatozoa are added after they have become inactive, the percentage of eggs that are fertilized depends upon the degree of inactivity and the degree of reactivation (such as is brought about by transferring spermatozoa to sea water without high hydrogen ion or carbon dioxide concentration) which the experimental conditions afford. The sooner spermatozoa become inactive and the more completely inactive they become, the longer their life. The hydrogen ion concentration of egg water that is most effective in preserving the life of spermatozoa is precisely that of acidified sea water that best subserves the same function.

Other effects of egg water upon the physiological condition of spermatozoa have not been considered in this investigation.

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